Department Mol. Biol
Subject 6/15/95 - 9/14/96
Name Ann Kim # 10
Address

National Brand
Computation Notebook
43-648
11¾" x 9¼", 4 x 4 Quad., 75 Sheets

0 73333 43648 8

Avery Dennison
Office Products
Chicopee, MA 01022

Ruben EXHIBIT #93

Department Mol. Biol
Subject 6/15/95 - 9/14/96
Name Ann Kim # 10
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National "Brand"

Computation Notebook

11¾" x 9½", 4 x 4 Quad., 75 Sheets 43-648

0 73333 43648 8

AVERY DENNISON
Office Products
Chicopee, MA 01022

6/23/95

Inoculate 100ml LB+Amp Kan
3ml A2 uninduced
Incubate 37°C until OD_{600} = 0.4-0.6
2½ hours w/aeration
Add 100mM IPTG to 2mM
2ml
Incubate 37°C w/aeration 4hours
Spin culture 10 min 3K.
Resuspend pellet 10ml 6M GnHCl pH8
Store 4°C over weekend

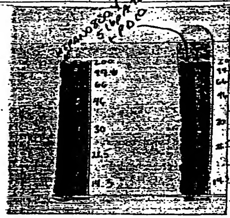
6/26/95

Isolate H TPAN08 S04 5t bp ATG+PDIO
in 6M Gn HCl pH5 in Acrylamide
preparative Gel.

in 430ul H_2O
Add 50ul Protein
50ul 0.15% Na-DOC
75ul 50% TCA
mix well
Spin 10 min
Resuspend pellet in 25ul 0.2N NaOH.
Combine into 2 Tubes
Add equal Volume ~~of~~ 2X Disassociation
Buffer
Heat 100°C 5 min
Run 100V 1 1/2 hours
Stain + Destain marker + part of gel
Cut desired band from gel
Cut up gel slice - ready to ship

6/26/95

pg 14] ~~~ ~~~



Send for Ab production to Pocono farm Cindy Hask
~0.75mg Acrylamide
~0.5mg eluted Imidazole

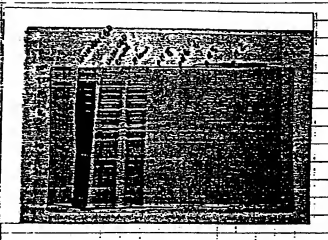
Spin Crude extract of HMSAF22-A2
8K 20 min
Transfer Supernatant to fresh tube
Prepare $NiSO_4$ Column
2ml Resin to make 1ml bed
Wash Column 10ml H_2O
Equilibrate with 20ml 6M GnHCl pH8
Add supernatant. Collect Flow
Wash column 30ml 6M GnHCl pH8
Collect pH8
Wash Column 30ml 6M GnHCl pH6
Collect pH6
Elute Protein 5ml 6M GnHCl pH5
collect eluted
Strip Column 30ml 6M GnHCl pH2
Collect pH2

Prepare to run on 12% Stacking PAGE Gel

6/26/79

430 µl H_2O
50 µl Protein
50 µl 0.15% Na DOC
75 µl 50% TCA

Mix well
Spin 10 min
Remove Supernatant
Resuspend pellet 20 µl 0.2N NaOH
Add 20 µl 2x Dissociation Buffer
Heat 100°C 5 min
Run 20 µl on gel in Mini Mighty
150V 1½ hrs
STAIN / DESTAIN



Doesn't look like anything indicated

Remake insert fragment

PCR Fragment	
11749 + 11848	20 µl
10x #2	6
10x BSA	6
H_2O	27
Xba I	1
	60

Incubate 37°C Overnight

6/27/95

Inoculate 5ml LB+ Amp + Kan
with frozen Stock of
HTPAN08 51bp + PD10 D5
from frozen Glycerol Stock.
Incubate 37°C w/aeration 2½ hours.
Inoculate 5ml LB+ Amp/Kam w/culture
Add 100ul 100mM IPTG to Culture
Incubate all 37°C w/aeration
Inoculate 50 ml LB+Kan/Amp
to do large Scale Induction

PCR Fragment.
Xba Digested.

PPt with Ethanol and NaAcetate
Spin 10min
Remove Supernatant
Wash pellet 70% Ethanol
Spin 5min
Remove Supernatant
Allow pellet to dry
Resuspend pellet / 44ul TE
Add 5ul Buffer M
1ul Sph I
Let Digest 37°C O/N

6/28/95

Inoculate 600 ml LB+Amp/Kan
with 35ml overnight Culture
of HTPAN08 504 51bp ATG + PD10
D5
OD_{600} ≈ 0.177
Incubate 37°C w/aeration for 2hrs
Until OD_{600} ≈ 0.4-0.6

@ 6/28/95

OD_{600} = 0.45
Add 100mM IPTG to 2mM - 12ml
Incubate 37°C with Aeration
4 hours
Spin cultures 4K 20min
Pour off supernatant
Resuspend pellet in a total of
80 ml 6M Gn HCl pH8
Store 4°C

Run Gels for Westerns
12% PAGE Gels - Stacking
15 well combs

Samples: 1 HTPANO8SO4 51bp ATG + PD10 D5 - UNINDUCED
2 " " " " " - INDUCED
3 HMSAF22 + PQE70 H10 UNINDUCED
4 HTPANO8SO4 51bp ATG + PD10 Purified pH5.
Run 15 μl each on gel

(3) (2) (1) - Spin 3ml Culture
Resuspend in 200 μl H_2O
Add 200 μl 2X Dissociation Buffer
Heat 100°C 5min
Spin 5min.
(4) - 900 μl H_2O
70 μl Protein in pH5
100 μl 0.15% Na DOC
150 μl 50% TCA
mix well
Spin 10 min
Resuspend pellet 150 μl in 0.2N NaOH
Add 150 μl 2X Dissociation Buffer
Heat 100°C 5min

6/28/95

Run -

3X
1 - Rainbow Marker
2 - HTPHNO8S04 .5 bp ATG + PD10 UNINDUCED
3 - " " " " " INDUCED
4 - HHSHF22 + PDE UNINDUCED
5 - HTPHNO8S04 .5 bp ATG - pH5.

Run 150 V 1.4 hours.

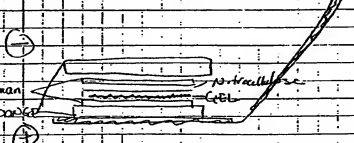
Set up Transfer.

Transfer Buffer:

25 mM Tris pH 8.3
192 mM Glycine
20% METHANOL.

4°C

Set up Sandwich



Run 100 mV
140 min
Run 200 mV
20 min

Store blots in Blocking Buffer

WESTERN BLOCKING BUFFER:

3% BSA Fraction V
0.02% Na AZIDE
in PBS

Ot 4°C

6/29/95

Run Gel of Fragment with 1Kb ladder

fragment looks good

Set up ligations at 24°C

3' Xba/5' SphI

	1	2	3	4	5	6
DNA Fragment	2	2	2	—	—	—
PQE70 NcoI/SphI	1	—	—	1	—	—
PQE70 SphI/Nco	—	1	—	—	1	—
10X Buffer	2	2	2	2	2	2
T4 ligase	1	1	1	1	1	1
H_2O	14	14	15	16	16	17

Incubate reactions 16°C overnight

HTPAN08504 81bp ATG + PD10 D5 protein

Spin 8K 30 min

Transfer supernatant to fresh tube

= Crude Extract

Prepare $NiSO_4$ Column

6/29/82

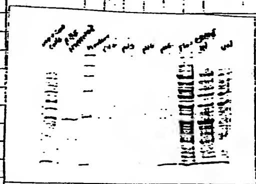
Prepare Bed - 3 ml
Strip Column - 30 ml 0.2N NaOH
Wash 50 ml H_2O
Charge 50 ml 0.1 M $NiSO_4$
Wash 50 ml H_2O - Equilibrate 30 ml 6 M Gn HCl pH
Add Supernatant to column
Collect - flow
Wash - 40 ml 6 M Gn HCl pH8
Collect - pH8
Wash - 40 ml 6 M Gn HCl pH6
Collect - pH6
Elute - 10 ml 6 M Gn HCl pH5
Collect - pH5
Strip - 40 ml 6 M Gn HCl pH2
Collect pH2

Run fractions on Gel

450 µl H_2O
50 µl Fraction
50 µl 0.15% NaDOC
75 µl 50% TCA

Mix well
Spin 10 min
Remove Supernatant
Resuspend pellet 10 µl 0.2N NaOH
Add 10 µl 2X dissociation Buffer
Heat 100° C 5 min
Spin 5 min @
Run all on 12% Stacking PAGE Gel 150 V 1 hr
Stain 30 min / DESTAIN 30 min

6/30/95



looks like most of the protein did not stick on the column —
Try reapplying crude extract (Flow thru) on a column with freshly charged resin.

Transform ligation

Thaw Chemically competent M15 cells on ice
To 100ul thawed cells add 10ul of ligation
Let sit on ice 1 hour
Heat 42°C 45sec
Put on ice 2 min
Add 400ul LB to tube
Incubate 37°C 1 hour
plate 100 ul onto LB+Amp/Kan plates

Incubate at Room temp over weekend.

7/3/95

Recieved primers for HTPAN08S04 into pCDNA 3' HA TAG
5' HA TAG
+ NoTag

#12305 HTPAN08S04
5' Bam HI + 5' Untranslated Region for 3' HA Tag in PCDNA.

BamHI
GCG GGC GGA TCC TGC CTG GCT GAGC TTA CAG CAGTC

#12306 HTPAN08S04
3' Xho I for 3' HA TAG in PCDNA
No Stop Codon

XhoI
GCG GGC CTC GAG GCC AAC TAA AAA GGC CCC GAA AAA ACT G

#12307 HTPAN08S04
5' Bam HI for 5' HA Tag and Regular pCDNA

BamHI
GCG GGC GGA TCC GCT ATG ATG GAG GTC CAG GGG GGA C

#12308 HTPAN08S04
3' Xho I for 5' HA Tag and regular pCDNA
has authentic stop.

XhoI
GCG GGC CTC GAG TTA TTA GCC AAC TAA AAA CGC CCC GAA AAA AC

PCR inserts

7/3/95

Set-up PCR

3' HA TAG		10X	5' HATAG		10X
12305	6	60	12307	3.7	37
12306	6	60	12308	6	60
10X dNTP	10	100	10xdNTP	10	100
10X PCR	10	100	10XPCR	10	100
TAQ	0.4	4	Taq	0.4	4
H_2O	66.4	664	H_2O	68.9	689
DNA	1	10	DNA	1	10
	100 μl			100 μl	

PCR.

95°C 5min
95°C 30 sec
55°C 30 sec } 25X
72°C 1/2 min
72°C 9 1/2 min
4°C Hold

Run 1% agarose gel

looks good
Combine Tubes

Precipitate with equal volume PEG/NaCl
Spin 10 min
Remove supernatant
Wash pellet 1000 μl 70% Ethanol
Spin 5 min
Pour off supernatant
Allow pellet to dry at RT
10 min

7/3/95

Resuspend pellet in a total of 100 µl TE
Run 1 µl on gel

HTPA001 SO4

Store -20°C Fragment #3 Box

(CV) 10

Pick colonies from transformation (6/30) into 1B7 Amp/Kan - HMSAF22

- 46 of #1 - 2 of #3
- 46 of #2 - 2 of #5

Incubate 37°C w/aeration 4 hours
Set up PCR

		10X
5' PQE Vector	0.2	20
3' PQE Vector	0.14	14
10X dNTP	3.2	320
10X PCR	3.2	320
H_2O	23.1	2310
Taq	0.16	16
Culture	2	
	32 µl	3000 µl - 30/tube

		100X
5' SphHMSAF22	1.6	160
3' PQE Vector	0.14	14
10X dNTP	3.2	320
10X PCR	3.2	320
H_2O	21.7	2170
Taq	0.16	16
Cult.	2	
	32	300 µl 30 µl/tube

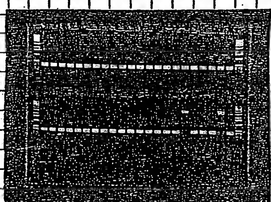
7/3/95

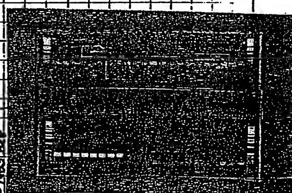
PCR Program #66

95°C	5 min	
95°C	20 sec	30X
55°C	20 sec	
72°C	1 min	
72°C	7½ min	
4°C	Hold.	

7/4/95 Holiday

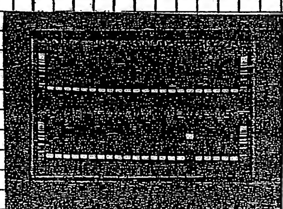
7/5/95 Run 10 ul of PCR reaction with 1KB ladder - HHSAF22



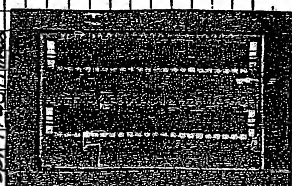


5' Specific

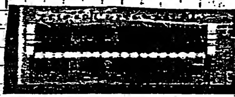
5' PDE + 3' PDE Vector Specific



3OH + PDE Primed



7/5/95



Does not look like the Ligation Worked

Try Retransforming Ligation Again
6/7/95

Set up Digestion of H1PAN08504 for pcDNA (pg 39)

5' HA TAG		3' HA TAG	
Fragment	20	Fragment	20
10X #2	5	10X #2	5
Bam HI	1	Bam HI	1
Xho I	1	Xho I	1
H_2O	23	H_2O	23
	50		50

Incubate 37°C Overnight

7/6/95

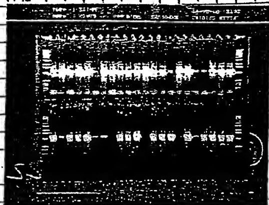
Pick more colonies from 6/9/95 - HMSAF22 into LB+ Amp/Kan
Incubate 37°C 4 hours
&
Set up PCR.

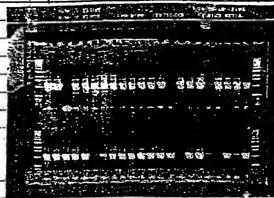
		Mdx
5' Sont	1.6	160
3' PQLS	0.14	14
10x dNTP	3.2	320
10x PCR	3.2	320
Taq	0.16	16
H_2O	21.7	2170

07/6/95

PCR Prog 66. HMSAPZ2

Run 10 µl on Gel w/ 1Kb ladder



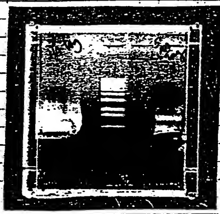


looks good.
Inoculate 200 µl of
LB + Amp/Kan
with (+) and 10 A(12)
Incubate 37°C w/
aeration overnight

LMP Gel Purification
HTPAD05SAP 3' HA TAg
5' HA Tag

Load onto 0.8% LMP Gel
Cut out gel slice
& Take Picture
Gene Clean fragment

HCPANα8SOY 7/6/95



Gene Clean
Add 1000μl NaI
Heat 55°C 5min
Add 10μl Glass Milk
Let incubate at RT 5min
Spin 10sec
Remove Supernatant
3X [Resuspend pellet in 500μl Wash Buffer
Spin 10sec
Remove Supernatant]
Spin 10sec
Remove Supernatant.

Resuspend pellet 40μl TE
Heat 55°C 5min
Spin 1min
Transfer Supernatant to clean tube
Resuspend pellet 20μl TE
Heat 55°C 5min
Spin 1min
Transfer Supernatant
Run 1μl of Fragment on gel with 1Kb ladder
Store -20°C in Fragment #3 Box.

HCPANα8SOY

Will need Vector to do ligations
None premade w/ correct Digestions. So set up Digests

pcDNA 5 HA	10μl	pcDNA 3 HA 6#	17
10x #2	5	MY	5
H_2O	33	H_2O	26.0
Bam / XhoI	1/1	Bam / Xho	1/1
	50		50

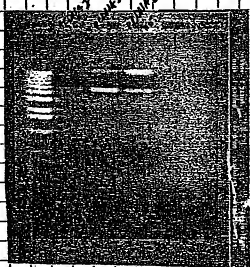
7/6/95

pCDNA I	1.1
10X #2	5
H_2O	41.9
BamHI	+ 1.1
	50

Incubate 37°C overnight

7/7/95

Ran 1 ul of Digest on Gel with 1Kb ladder



pCDNA should be ~4.7 Kb

pCDNA 5' 45 HA Tag ~~[illegible]~~
Sal I DNase insert ~600 bp.
pCDNA I No inserts

looks like not completely digested.
Add 1 ul more Enzyme. Incubate 37°C. [illegible] [illegible] Day
[illegible] 37°C over weekend.

Rec'd Clone from Susie

Clone I.D. HE2PM 21 # 2 (RB)
Requested By Ann Kim
Transfer Folder 07/05/95 transfer 2
Processed By Susie Riedorf

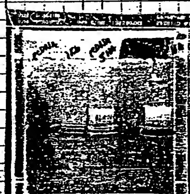
[signature] 7/7/95

7/7/95

Small scale inductions
- from overnight cultures from pg 42.
Inoculate 700 ul LB + Amp Kan with 15 ul of culture -
Incubate 37°C w/ aeration 2hrs.
OD_{600} = 0.4 - 0.6
Add 100mM IPTG to 2mM - 4ul
Incubate 37°C w/ aeration 4 hours
Spin culture 10min
Remove supernatant
Resuspend pellet in 10 ul H_2O
Add 10 ul 2x Dissociation Buffer
Heat 100°C 5min
Spin 2 min
Run 5 ul on gel with marker and uninduced culture on 15 well
of 12% Stacking gels.
Run 150V 1.3 hrs
Stain over weekend at RT.

7/10/95

Run pCDNA Digests on 0.8% LMP Gel
with 1 kb ladder



Cut out Gel Slices
Gene Clean

Resuspend Gel Slice
800 ul NaI
Heat 55°C 5min
Add 5 ul Glass milk
Let sit at RT 2-3 min
w/ occasional mixing
Spin 10 sec

7/10/95

Remove Supernatant
Resuspend pellet 100µl Wash Buffer
Spin 10 Sec
2X { Remove Supernatant
Add 100µl Wash Buffer
Spin 10 Sec }
Remove Supernatant
Spin 10 Sec
Remove supernatant
Resuspend pellet 15µl TE
Heat 55°C 1 min
Spin 10 Sec
Transfer Supernatant to fresh tx tube
Resuspend pellet 15µl TE
Heat 55°C 1 min
Spin 10 Sec
Transfer Supernatant to New tubes
Run 2µl on gel with 1Kb ladder

Vectors look good
Store

Store -20°C
Vector #2 B22

Set up ligations
with HPRANO8SO4
5' + 3' HA Tag
Fragments
(pg 48)

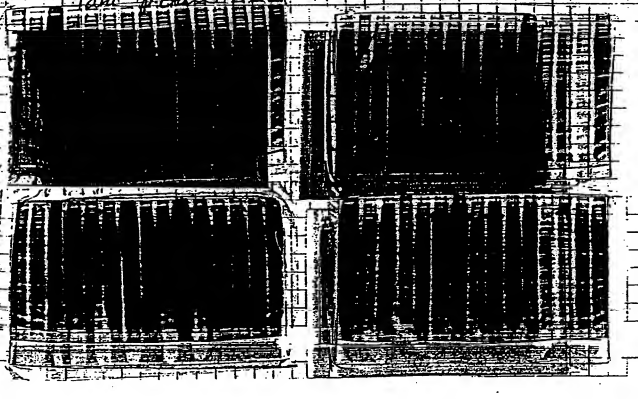
7/10/95

	1	2	3	4	5	6	7	8	9
HTOANOR504 5' HA Tag 6um/kb	1	1		1					
HCOANOR504 3' HA Tag 6um/kb			1		1				
pCDNA 6um/kb	4					4			
pCDNA 5' HA 6um/kb		3					3		
pCDNA 6um/kb+3' HA			2	1				2	
12% Buffer	2	2	2	2	2	2	2	2	2
H_2O	13	[illegible]	14	16	16	13	14	15	17
Ty Ligase	1	1	1	1	1	1	1	1	1

Incubate 16°C overnight

DE STAIN Gels — 37°C — 2 hrs. — HMSA#22 PDE70

Take pictures



7/11/95

	5'HA	3' HA	pCDNA
DNA	17	27.8	1.8
10X Buffer	5	5	5
10X BSA	5	5	5
H_2O	22	11.2	37.2
Enzyme	1	1	1
	50	50	50 ul

Digest with Xho I and BamHI

Incubate 37°C O/N.

HTPAN08 SO4 pCDNA constructs.
ligation from 7/10/95 pg 47

Transform ligations (10ul) into 100ul of Chemically Competent DH5α cells.

Thaw Cells on ice
Aliquot 100 ul into Sterile tubes
Add 10 ul of ligation
Mix by pipetting
Incubate on ice 1 hour
Heat 45°C 45 Sec
Place on ice 2 min
Add 400 ul LB
Incubate 37°C 1 hour
Plate 100 ul + 200 ul onto LB Amp plates
Incubate 37°C overnight

7/12/95

Pick transformed cells into LB+Amp H1PAN08504 + pCDNA construct

1 H1PAN08504 + pCDNA
2 H1PAN08504 + 5'HA Tag pCDNA
3 H1PAN08504 + 3'HA Tag pCDNA

Incubate 37°C w/ Overnight 4 hrs
Set up PCR.

for #1&2		for #3	
H2307	2	12305	2.5
FPB/3	DDI	FPB	0.01
10xdNTP	3.2	10xdNTP	3.2
10xPCR	3.2	10xPCR	3.2
Taq	0.15	Taq	0.15
H_2O	21.9	H_2O	20.9
Culture	2	Culture	2
	32		32

PCR Prog 66

A. H1PAN08504 plasmid
B. H_2O only

95°C	5 min	
95°C	20sec	30x
55°C	20sec	
72°C	4 min	
72°C	7½ min	
4°C	Hold	

Run 10µl on Gel with 1Kb Ladder

No Positives seen - Not even (+) plasmid PCR'd. Redo Tomorrow with fresh primers

Cassie Out 07/13-07/17

7/13/95

Add Equal Volume PEG/NaCl
4°C O/N

PCR.

H7PAN08 - PC DNA Constructs

1&2   3

12307	2	120305	3
12308	3	12306	3
10X dNTP	3.2	10X dNTP	3.2
10X PCR	3.2	10X PCR	3.2
H_2O	18.4	H_2O	17.4
Taq	0.2	Taq	0.2
Cutkur	2	Cult	2
	32		32

Run PCR Prog Col. 48 (pg 57 for gel picture)

Galacto Kinase Assays:

D(+) Galactose + ATP $\xrightarrow{GalK}$ α-D-Galactose-1-phosphate + ADP

ADP + PEP $\xrightarrow[Mg^{++}/K^{+}]{PK}$ Pyruvate + ATP

Pyruvate + β-NADH $\xrightarrow{LDH}$ Lactate + β-NAD

OD_{340}

Reagents.

A: 160 mM Potassium Phosphate Buffer pH 7.0

B: 100 mM D-Galactose

C: 59 mM Adenosine 5' Triphosphate (ATP)

7/13/95

Add 13% PEG 8000 / 1.6 M NaCl - 850 µl
Mix well.
Store -20 °C O/N

Ratio of Galacto [illegible]

[illegible]/95

T=24, 30°	30-180 sec	T=0	30-180 sec	T=24, 4°C	30-120 sec
1-1	0.6582 - 0.6581 / 150 sec; -6×10^{-6}/sec	1-2	0.6919 - 0.67152 / 150 sec; 1.1×10^{-4}/sec	1-3	0.7787 - 0.7814 / 90 sec; -3×10^{-5}/sec
2-1	0.6665 - 0.5709 / 150 sec; 6.4×10^{-4}/sec	2-2	0.6064 - 0.4669 / 150 sec; 9.3×10^{-4}/sec	2-3	0.8018 - 0.7768 / 90 sec; 2.8×10^{-4}/sec
3-1	0.7086 - 0.7033 / 150 sec; 3.5×10^{-5}/sec	3-2	0.6877 - 0.6704 / 150 sec; 1.3×10^{-4}/sec	3-3	0.7730 - 0.7730 / 90 sec; $-5.5 \times 10^{-?}$/sec
4-1	0.6825 - 0.5931 / 150 sec; 6×10^{-4}/sec	4-2	0.6203 - 0.4792 / 150 sec; 9.4×10^{-4}/sec	4-3	0.7854 - 0.7587 / 90 sec; 3×10^{-4}/sec
5-1	0.6841 - 0.5678 / 150 sec; 7.8×10^{-4}/sec	5-2	0.6273 - 0.4916 / 150 sec; 9.0×10^{-4}/sec	5-3	0.9937 - 0.7885 / 90 sec; 5.8×10^{-5}/sec
With Gal K	0.6105 - 0.1350 / 150 sec; 3.2×10^{-3}/sec	Blank	0.7250 - 0.7212 / 150 sec; 4.5×10^{-5}/sec	Acetate Gal K+ 10% Gly	0.533 - 0.1695 / 90 sec; 4×10^{-3}/sec

7/14/95

Ri-PCR HPAL 208 S04 pCDNA Constructs
Run on gel with 1 Kb ladder

See pg 54

7/17/95

HS/HQ DT -20° 7/14/95	0.9765 − 0.6006 / 150 sec 1.2×10⁻³/sec	HQ DT HS -20° 7/14/95	0.8019 − 0.6740 / 150 sec 8.5×10⁻⁴/sec
Gal +10% Glyc -20° 7/13/95	0.6771 − 0.1012 / 150 sec 3.8×10⁻³/sec	Blank	0.8696 − 0.8653 / 150 sec 2.9×10⁻⁵/sec

Inoculate 5ml TB+Amp
with HTPANO8 pCDNA Cultures

1: A1, A2, A3, A4
2: D1, D2, D3, D4, E1, E2, E5
3: E9, E10, E2, G1, G2, H3, H4

Incubate 37°C w/ aeration O/N

7/18/95

Boiling mini preps - HTPANO8 pCDNA Construct

Spin 5ml culture
Remove Supernatant
Resuspend pellet 800µl STET + RNase + Lysozyme
Heat Samples 100°C 1 min
Spin 5 min
Remove pellet
Add 800µl 13% PEG 8000/1.6M NaCl - mix well
Spin 10 min
Remove Supernatant
Wash pellet 1 ml 70% Ethanol
Spin 5 min

7/18/95

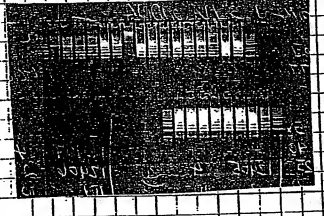
Remove Supernatant
Wash pellet to dry at RT 10 min
Resuspend pellet in 250 ul TE
Run 1 ul on gel with 1 kb
Add

Set up Digestion

	Vol	Rxn
		10X
DDH	10	
Cut2	3	54
H2O	10.8	302.4
Buffer	0.1	1.8
Mix	0.8	1.8
	30	

Incubate Digests at 37° O/N

Run HHUSAER27 + POE Digests on 1% Gel with 1 kb ladder - Bgl II / Sph I



Cannot tell anything with this digest
- Too much Chromosomal
- Try Digests with
- EcoRI and HIII

9/19/95

Run [illegible] of fragment on gel with 1Kb ladder

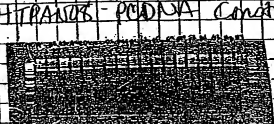
HG05H43 T7+ATG ready for TA / Store -20° Fragment #2 Box.

HT45B02 Fragments Set up Digest

3'HA 12408+12409				5'HA 12406+12407				PA2 12404+12402				PQE 12403+12405			
DNA	10	DNA	10	DNA	10	DNA	10	DNA	10	DNA	10	DNA	10	DNA	10
10X	5	10X	5	10x	5	10x	5	10x	5	10x	5	10x	5	10x	5
H2O	34	H2O	34	H2O	34	H2O	34	H2O	34	H2O	34	H2O	34	H2O	34
Bam	1	Xho	1	Bam	1	Xho	1	Bam	1	Xba	1	Bam	1	Nco	1
	50		50		50		50		50		50		50		50

Incubate 37°C 4 hrs - Add Alternate enzyme and incubate ON at 37°C

HTRAN08 - PCDNA Constructs - Bam/Xho Digests



7/20/95

HTPA1008 Midi preps

Spin 5 ml culture
8K 15 min
Resuspend pellet in Resuspension Buffer + RNase + Dnase
Add 100 ml Lysis Buffer
Mix Gently
Let sit 10 min at RT
Add 10 ml Neutralization Buffer
Mix well
Let sit on ice 20 min
Spin 8K 30 min
Transfer Supernatant to fresh tube
Add 10 ml 30% PEG and 10 ml 5 M NaCl
Mix well
Store 4°C 20 min
Spin 8K 30 min
Pour off Supernatant
Resuspend pellet in 10 ml 70% Ethanol
Spin 8K 15 min
Pour off Supernatant
Allow pellet to dry slightly
Resuspend in 2 x 400 µl TE
precipitate DNA
1/10 Vol 3M NaAc + 2x Vol 100% Ethanol
Store -20°C O/N

7/21/95

7/22/95

Pick Colonies into LB + Amp Media

1-24	6-12	9-48
2-24	6a-12	10-96
3-12	7-24	
4-12	8-24	

7/21/95

Clones look good –
Monday ... inoculate for minipreps.

HTPANO8

Spin DNA 10 min
Pour off Supernatant
2X (Wash pellet 2X 70% Ethanol
Spin 5 min
Pour off Supernatant
Let pellet Dry at RT
Resuspend in 400 ul of TE Total
Ready to clean-up

7/24/95

Inoculate 5 ml TB + Amp w/
Cultures from HT4SB02 Ligation

1	HT4SB02	3' HA TAG B/X	A1, A2, A3, A4
2		3' HA TAG X/B	C1, C2, C3, C4
3		5' HA TAG B/X	E1, E2, E3, E4
4		5' HA TAG X/B	E5, F6, F7, F8
5		pCDNA B/X	G1, G2, G3, G4
6		pCDNA X/B	H5, H6, H7, H8
7		PA2 B/X	A1, A2, A3, A4
8		PA2 X/B	C8, C10, C12, D1

+ pCDNA 3' HA Tag — 15
pCDNA 5' HA Tag — 16
pCDNA — 17
PA2. — 18

Incubate 37°C with aeration
over night

+B = 1
+D = 2
= 3
= 4
= 5
1-6
pCDNA
3 = 8
D = 8
ontrols
ector
lone
PA2
1/2
trols

7/24/95

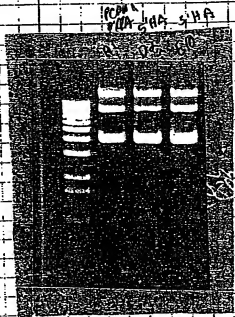
Transform ligations from 7/20/95
ligations Pg 70 #9 to 10 - HT48802 + PQE60

Thaw M15 rep4 cells on ice
Add 100ul of chemically competent cells to 10ul of ligations
Incubate on ice 1 hour
heat 42°C 45 sec
Place on ice
Add 400ul LB
Incubate 37°C 1 hour
Plate 100 & 250ul onto LBT Amp/Kan plates.
Incubate at 37°C O/N.

HTPAN08 pCDNA Constructs

2x Phenol/Sevag (1:1) extract
3x Sevag extract
ppt DNA
1/10 vol 3M Na Acetate pH 5.3
2 vol 100% Ethanol
Mix well
Spin 10 min
Pour off supernatant
Wash pellet 1000ul 70% Ethanol
Spin 5 min
Pour off supernatant
Allow pellet to dry at RT 10 min
Resuspend pellet 200ul TE
Run 1ul on gel with 1kb ladder.

7/24/95



plasmid looks good

Set up Digests

DNA	4
10X #2	3
H_2O	22
Bam HI	0.5
Xho I	0.5
	30 µl

Incubate 37°C O/N
Run on gel tomorrow.

7/25/95

RADIATION PROBLEM FOUND -
- UNABLE to travel freely around 3rd floor 9610

- Take plates out of 37°C
H-hSB02 pQE60

- Take culture tubes out of 37°C
H-hSB02 + pQE2 + PC DNA

- Boiling minis
Spin 2 ml centrifuge 5 min
Remove supernatant
Resuspended pellet 1000 µl STET + RNase and lysozyme
Heat 100°C 2 min
Spin 10 min

7/25/95
Remove pellet
Add equal volume 13% PEG/NaCl
Mix well
Store -20°C

7/26/95 NO WORK + Cleaning of
of Radioactivity by RSO.

7/27/95

Spin B. Minis 15 min
Remove Supernatant
Wash pellet w/ 5 ml 70% Ethanol
Spin 5 min
Remove Supernatant
Allow Pellet to Dry at RT 15 min
Resuspend in 300 µl TE
Run 2nd on gel with 1 kb ladder

1-8 HT4SB02 + 3'HA pCDNA
A1, A2 A3, A4, C1, C2, C3, C4
9-16 HT4SB02 + 5'HA pCDNA
E1, E2, E3, E4, F5, F6, F7, F8
17-24 HT4SB02 + pCDNA
G1, G2, G3, G4, H5, H6, H7
25-32 - HT4SB02 + PA2
A1, A2, A3, A4 C8, C10, C12 D1
33 - 3'HA pCDNA
34 - 5'HA pCDNA
35 - pCDNA
36 - PA2

7/27/95

Set up Digestions

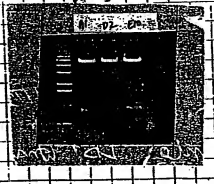
11-24 + 33, 34, 35 Digest Burn/Vhs

25-32 + 36 Digest with Burn/Vhs

DNA	1D	29X	DNA	1D	10X
10X #2	3	87	10X #2	3	30
H2O	1618	487.2	Burn #1	0.1	1
Burn #1	01	2.9	Vhs I	0.1	1
Vhs I	01	2.9	H2O	16.8	168
	30ul	20ul/tube		20ul	210ul/ea

Incubate at RT - 3 hrs

Run HTPAND Digests on gel w/ 1Kb ladder



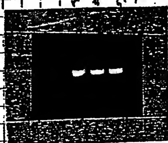
Digests look good

Submit for Seq. with internal primers

8/10/95

Run ..... of plasmid on gel w/ 1.4
plasmid looks good

HT4SB02



Setup Digests

Bam / XhoI

DNA	1
10X	3
H_2O	25.6
BamHI	0.2
XhoI	0.2
	30ul

Incubate 37°C O/N

Transfections

Remove Spent media
2X Wash Cells in 3ml DMEM without Serum

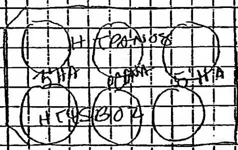
make transfection Cocktail
300 ml
or 3ml Nutridoma
0.75ml DEA Dextran (20mg/ml Stock)
0.8ml Chloroquin (5mg/ml Stock)
25.8 ml DMEM

To 4ml of each Transfection Cocktail
Add 10ug DNA

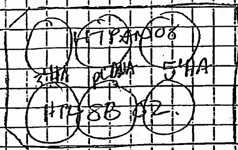
HT4SB02 pCDNA Constructs
HTIGN08 pCDNA Constructs

8/16/95

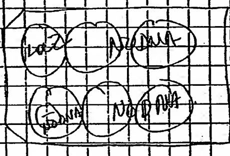
AMK COS



pl1 COS



AMK COS



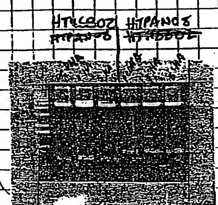
2ml per well
Incubate 37°C
2hours 5.0% CO_2

Remove Transfection Cocktail
Add 1ml ~~Glyc~~ Shock Media
DMEM + 10% FCS + 10% DMSO
Incubate 2.5 min - Time is critical
Remove Shock Media
Add 1ml DMEM
Remove Media
Add 3ml complete DMEM
Incubate 37°C for 72hrs
in CO_2 Incubator.

8/17/95

Run Digestion gel
w/ 1Kb ladder

looks good.
Submit HT4SB02
for Sequencing



8/30/

Digest with BamHI/XbaI - HEZAMZI+PAZ

DNA	2
H_2O	15.6
10x#2	2
BamHI	0.2
XbaI	0.2
	20ul

Incubate 37°C 2hrs
Run 10ul on gel w/ 1Kb ladder


HEZAMZI+PAZ
BamHI/XbaI

Submit for Sequencing with internal primers and with PAZ specific primers.

HTY5802 + PAZ
Construct sent to Protein Expression
Submit for Sequencing to Confirm

FAB Ligand - HTPAXXX
Antibody - Rec. Ab Rabbit Ab
from Pocono farms
Reading for Western Detection

FASLIG #1 RABBIT #111940 → #1
#111941 → #2

Pre Bleed - 6/30/95
Test Bleed #1 - 8/25/95

8/30/95

Western blots from 6/28/95

(See pg 31-32)

Remove 2 blots from Blocking Buffer

To 10 ml Fresh blocking buffer add 100 ul Ab Serum - 1:100 Dilution

Incubate O/N at RT Shaking

#1 = Test Bleed #1 - #11941
#2 = Test Bleed #1 - 11940
3 = Prebleed #11940
4 = Prebleed #11941

8/31/95

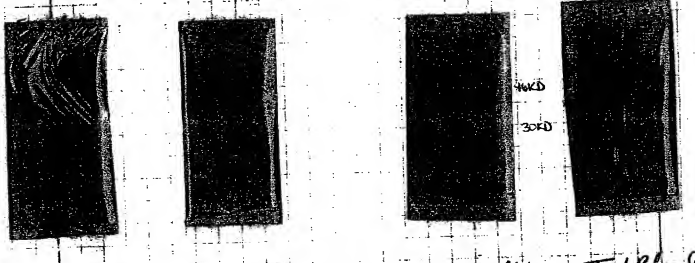
FAS LIG Western

Cont.
Pour off 1° Ab
Rinse filters in 1X PBS
Wash 1X in 10 ml 1XPBS 5 min at RT w/ Shaking
Add 2° Ab - Goat Anti Rabbit Peroxidase - Dilute 1:2000 in 1X PBS
Incubate at RT w/ Shaking for 1 1/2 hrs
Rinse filters 1XPBS
Wash 1X in 10 ml 1XPBS - 5 min at RT w/ Shaking
Rinse filters in 20 ml 50 mM NaH_2PO_4 pH 7 for 5 min at RT w/ Shaking

8/30/95

Add Substrate:
12.5 ml 50 mM $NaxH_2PO_4$ pH 7
25.0 mg β-NADH
25.0 µl - Phenol Equilibrated
or TRIS pH 7.0
8.5 µl 30% H_2O_2 Solution
375 µl NBT - Nitro Blue Tetrazolium
(10 mg/ml)

Incubate at RT till color change develops.
Stop Rxn with dI H_2O.
Dry and Label blots
FAS LIG - WESTERN.



Prebleed #11941 | Prebleed #11940 | 1° Ab. | Test Bleed #11940 | Test Bleed #11941

lane #1 - Rainbow Marker
#2 - HTPAN085C4 51bp ATG in PDID UNINDUCED
#3 - " " " " " INDUCED
#4 - HUSAF22 +PQE70 INDUCED
#5 - HTPAN085C4 51bp ATG in PDID - purified NTA Column - pH 5.0.

See Pg. 141

8/31/95

PCR HTPAN08 Constructs
PGE60

12975 5'Pr	2	12986 185bp	2
2865	0.4	2865	0.4
10x	10	10x	10
10x	10	10x	10
H_2O	76.3	H_2O	76.3
Taq	0.3	Taq	0.3
DNA 5ng/µl	1	DNA 5ng/µl	1
	100		100

PCR HE2PM21 +RA2 constructs

T7 Bac	0.1
128915 3' Bam I	2
10x	10
10x	10
H_2O	76.6
Taq	0.3
DNA 5ng/µl	1
	100

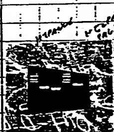
PCR Prog # 42.

95°C 5min
95°C 30sec ⎤
55°C 30sec ⎥ 25x
72°C 1min ⎦
72°C 7-12 min
4°C Hold

Total Volume PCR Rxn 500µl

8/31/95

Run 5ul of PCR product on Gel with 1Kb ladder



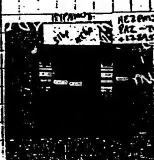
Add equal Volume of 13% PEG / 1.6M NaCl (500ul) per microcycle. Precipitate at 4°C O/N.

Inoculate 50ml c LB + Amp/Kan from colonies inductions
HYPAN08 51bp + 1K7 + PD10
HE2PM21 + POEG0 #1
Incubate 37°C w/aeration O/N

9/1/95

Spin DNA fragments 10min
1X 70% Ethanol Wash
Spin 5min
Pour off Super.
Allow pellet to Dry ~10min @ RT
Resuspend pellet in 100ul TE

Run 1ul on gel w/ 1Kb ladder



fragments are ready for digest

Submit HE2PM21/HPAZ TP +3 Kbs fragment for INT TO CPF

L Run

9/1/95

HTPAN08 5lbp A1G + PDIO
HE2PM24 + PQE60 #1

Inductions

To 300 ml LB+Amp inoculat
O/N culture till OD ≈ 0.8
Incubate w/ aeration at 37°C
for 2 hrs — till OD_{600} = 0.4-0.6

Add 100 mM IPTG to 2 mM ≈ 6.5 ml
Incubate with aeration 4 hrs
at 37°C
Spin 5K 15 min
Pour off Supernatant
Resuspend pellet in 10 M Tris HCl pH 8.
HE2PM24 ≈ 40 ml
HTPAN08 ≈ 20 ml
Store 4°C over weekend.

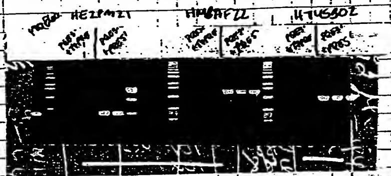
9/4/95 Labor Day
Off.

9/5/95

Spin Cultures 8K 20 min
Transfer Supernatant to fresh
tube
Prepare fresh Ni column
2 ml Resin Bed
20 ml 0.1 M Ni SO4
20 ml dH2O

9/5/95

Run 10 ul on gel with 1Kb ladder



looks like one of the primers is not working
- probably # 18496 - T7 pQE60
make new PQE60 T7 primer
& redo.

Digest New HTPAN08 SOU 51bp + 185bp
PQE 60 constructs

PCR Fragment	10	Incubate 37°C O/N.
10X	5	
H_2O	34.0	
Nco	0.5	
BglII	0.5	
	50ul	

9/6/95

Precipitate Digests
Add 150 ul TE - 200 ul 13% PEG/NaCl
Mix well
Spin 10 min

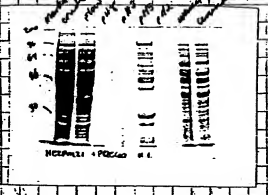
9/16/95

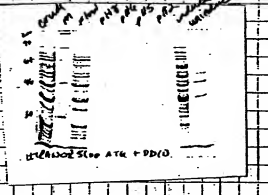
Wash pellet 70% Ethanol 500µl
Sonic | 5 min
Resuspend | Sonic instant
allow pellet to Dry at R.T - 10min
Resuspend pellet | in 10µl TE
Set up ligation

5Hp N/B	4	—	4	—	—	—	—
5Hp N/B	—	4	—	4	—	—	—
PstI N/B	2	2	2	2	2	2	2
10µ	2	2	2	2	2	2	2
Tot Digest	1	1	1	1	1	1	1
H2O	11	11	13	13	15	17	

Incubate 16°C O/N.

DE STAIN Gels





Computer Work

9/7/95

Transform ligations

Thaw DH5α cells on ice
100 ul of Chemically Competent cells add 10 ul ligation
Let sit on ice 1 hour
Heat 42°C 45 sec
Place on ice
Add 400 ul LB
Incubate 37°C 1 hour
plate 100 + 200 ul onto LB+Amp/Kan plates
Incubate 37°C O/N

- HTPAK78 51bp - Ab from Poconofarms → Block O/N
1:200 Dilution in Blocking Buffer

Computer Work -
ORF for HNFAA64
HTUSB02
HSEBN09
HMSAF22
HE2PM21

9/8/95

Pick Clones into 200 ul LB+Amp/Kan in 96 well dish
~90 of 185bp ATG and 51bp ATG
Incubate 37°C 4 hrs w/ aeration
Ready to Do PCR to Check for inserts

9/8/95

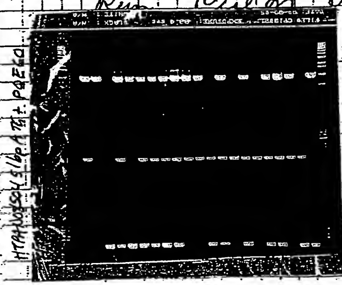
PCR to check for H7RANOBSOY
51bp & 185bp ATG

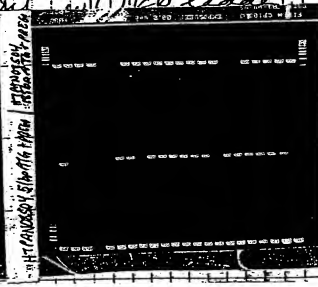
51bp		90x	185bp		90x
5' Bam Xho	2	180	5' Nco	2	[illegible]
3' [illegible] PAE	0.1	9	3' [illegible] PAE	0.1	[illegible]
10x	3.2	288	10x	3.2	28[illegible]
10x	3.2	288	10X	3.2	2[illegible]
H_2O	21.3	1917	H_2O	21.3	19[illegible]
Taq	0.2	18	Taq	0.2	18
Cult	2		cult	2	
	32			32	

PCR program 66

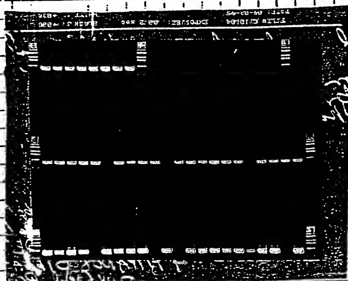
95°C 5 min
95°C 20 sec ⎤
55°C 20 sec ⎥ 30X
72°C 1 min ⎦
72°C 7 ½ min
4°C Hold

Run 10 ul on gel w/ 1kb ladder





9/8/95



HTPAN0804 185+ ATG + PQE60

90 +
180
9
288
288
1917
18

Looks like transformations worked very well.

A–H = HTPAN08<
51bp+ ATG + PQE

A'–H' = HTPAN0804
185bp ATG + PQE60

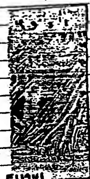
Inoculate 3ml LB + Amp / Kan with 10ul of 4hr culture.
Incubate at RT over weekend
1–26 of both 51bp + 185bp ATG.

FAS Ligand AB. See pg 8/31/95 (P1 131)
#11940 & #11941 large bleed

Pour off Buffer + 1° Ab.
Wash 1X PBS 10 min.
Add Anti Rabbit Peroxidase (2° Ab)
at 1:2000 in 1x PBS
Incubate at RT w/ shaking 1½ hrs
Wash filter 1x PBS 10 min
Rinse 50mM $Na_2H_2PO_4$ pH 7 for 5 min
Add Substrate: 12.5ml 50mM $Na_2H_2PO_4$ pH 7
25 mg β-NADH
25ul Phenol
8.5ul 30% H_2O_2
0.375 ml NBT

9/8/95

Shake until color Appears to desired Darkness.
Stop Reaction by adding dH_2O
Rinse filters 2x ddH_2O
Dry on Whatman paper



lane
1 HTPAN08 51bp ATG-Un
2 HTPAN08 51bp ATG-Ni
3 HUSAF22 + PGE70 - 1x
4 HTPAN08 51bp ATG purified Protein

9/11/95

To the cultures left at RT over the weekend add 100mM IPTG to 2mM - 10ul
Incubate 37°C 4 hours
Spin 200ul Culture
Resuspend pellet 75ul H_2O
Add 75ul 2x dissociation Buffer
Run 12ul on 10% Gel with Rainbow Ladder

9/11/95

Run 100V 1½ hours

STAIN Overnight

#13101- HEZPM21 5' BamHI-PA2
GCG CGC GGA TCC GCC ATC ATGGCGGCAGCA GTGGTGC

PCR New HEZPM21 PA2 Construct
with New 5' Bam primer
#13101

13101	2
3' Xba	2
10x dNTP	10
10x PCR	10
H_2O	74.8
Taq	0.2
DNA (10ng/µl)	1
	100

total of 100µl Rxn.

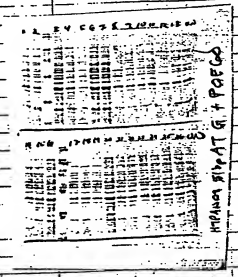
PCR Prog 75

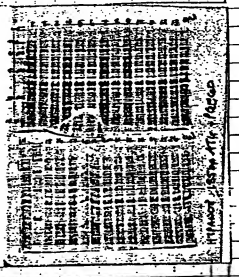
95°C 5min
95°C 30sec
55°C 30sec } 25x
72°C 1.5min
72°C 7.5min
4°C Hold

9/12/95

DESTAIN HTPANC8 Gels

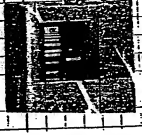
9/19/95





Looks like HTPAN08 185 bp ATC induced
Put Wat 5/bp
Do Boiling prep + Digest
Inoculate 5 ml TB + Amp/Kan
with 1:18 of 5/bp + 185bp
Incubate 37°C O/N Isolate

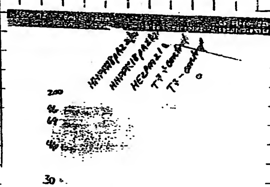
HE2PM21 PA2 Construct
Run ID on gel with
1Kb ladder



looks good
PPT with equal Volume
13% PEG / NaCl
Spin 10 min
Pour off Supernatant
Wash pellet

9/12/95

	TNT RESULTS 9/12/95		
INVESTIGATOR	SAMPLE NAME	EXPECTED PRODUCT SIZE KDa	OBSERVED PRODUCT SIZE KDa
MARIO CEPEDA	HTSFH75	30 OR LESS	NO PRODUCT OBSERVED
LAURIE INSCORE	HHFFK18	44	48 (major product), 44,31,23
LAURIE INSCORE	HHFFK18 PA2 2/25	33	NO PRODUCT OBSERVED
LAURIE INSCORE	HHFFK18 PA2 3/18	33	NO PRODUCT OBSERVED
ANN KIM	HE2PM21 PA2	44	36 (faint band)
T7 POSITIVE CONTROL	DNASE 02-105	33	33
T7 NEGATIVE CONTROL	WATER	NONE	NONE
T3 POSITIVE CONTROL	HCACI93	33	33
T3 NEGATIVE CONTROL	WATER	NONE	NONE



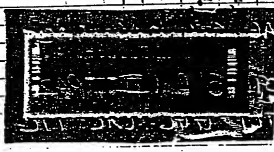
Set-up PCR of New TNT Constructs using the D 12aw T7 PQE 60/70 primer (See pg 136 9/5/95).

HE2PM21		HMSAF22		HT4SB02		HTPAN	
#4042	0.5	4042	1.5	4042	1.5	4042	
3' Xba	1.5	3' Bam	1.5	3'	2.5	3'	
10x dNTP	10						
10 x PCR	10						
H2O	76.5						
Taq	0.13						
DNA	1						

9/12/95

PCR Prog 66

Run 10µl on gel w/ CKbladder



looks like only HmSAF22 Worked Well - Try different program?

T7 Promoter is very long #4042 - 39mer

PEG ppt HmSAF22

9/13/95

To Rxns from 9/12/95

HE2PM21		HT4SB02		51bp HTPAW08		185bp HTPAN08	
4032	0.1	4032	0.1	4032	0.1	4032	0.1
3'	1.5	3'	1.5		1.5		1.5
10x	1	10x	1		1		1
10x	1	10x	1		1		1
H_2O	6.1	H_2O	6.1		6.1		6.1
Taq	0.3	Taq	0.3		0.3		0.3
	10		10		10		10

10µl / Tube

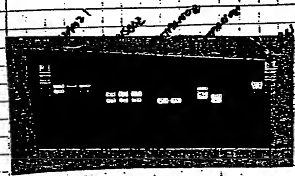
70

TPAN

42

9/13/95

Run 10 µl on Gel w/ 1Kb ladder



All PCR Runs seem to have worked

PEG PPT
- Add Equal Vol PEG / NaCl
- 70% Ethanol Wash
- Reppt 50 µl TE

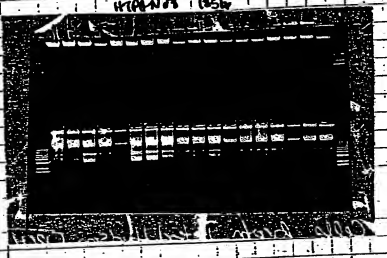
GET Cos Cells from Peter Hudson (PCH) frozen Stock - Plate onto T25 flask

Boiling mini Prep.

Spin 2ml culture 5 min
Resuspend pellet 750 µl STET + RNase / Lysozyme
Boil 5 min
Spin 10 min
Remove Pellet
Add 750 µl PEG/NaCl
Mix Well
Spin 15 min
Remove Supernatant
Wash Pellet 70% Ethanol 1 ml
Spin 5 min
Remove Supernatant
Allow Pellet to Air Dry 15 min
Resuspend pellet 200 µl TE
Run 2 µl on gel w/ 1Kb ladder

150

9/13/95



Set up Digests:

		36X
DNA	5	10Z
10x	3	7.7.6
H$_2$O	21.6	7.2
Nco I	0.2	7.2
Bgl II	0.2	
	30µl	

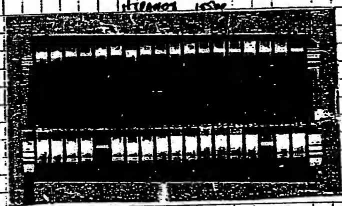
Incubate 37°C O/N

9/14/95

Run 2nd 06 PPH PCR fragments in Gel w/ 1kb ladder

9 9/14/95

Run Bailey Mini prep Digests on 1% gel with 1Kb ladder - 10 ul



Nco/Bgl II Digest

All look like they digested correct

Should induce

Run Westerns of 51 bp induction (see pg 144 9/12/95)

Run 150 V

Transfer blot 100V 1hr.

Block 2 hours with Blocking Buffer

Pour off

Add 1° Ab at 1:200 - Fab Lig

4th Bleed # ~~[illegible]~~ 1149 His Ab

Binding Buffer

9 Incubate O/N w/ shaking

See pg 3 Book #11 Lab Note book 405